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(54) Title: QUANTITATIVE TESTING FOR VITAMIN B12

(57) Abstract

The vitamin B12 (cobalamin) level of human blood and mammalian tissue is routinely assayed utilizing radioisotope dilution assay (RIDA) techniques. The presence of vitamin "B12 analogues" in such samples, which analogues are erroneously assayed as true vitamin B12 utilizing prior art RIDA techniques, has been determined. Such errors are due to the fact that binding proteins commonly used in prior art RIDA techniques normally include proteins which bind both true vitamin B12 and previously unrecognized vitamin B12 analogues. The errors caused by the B12 analogues are avoided by using a composition for binding vitamin B12 which is substantially free of substances which bind vitamin B12 analogues. Moreover, by using both types of binders in separate assays, the amount of analogue present may be arrived at by taking the difference of the assays.

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QUANTITATIVE TESTING FOR VITAMIN B_{12}

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods and materials

5 for assaying mammalian blood and tissue. More specifically
it relates to methods and materials for determining the amounts
of vitamin B_{12} and vitamin B_{12} analogues in human plasma.

Prior Art

For many years it has been recognized that the assay of

10 the vitamin B_{12} level in humans is a valuable technique for
diagnosing and subsequently treating certain diseases, such
as for example, pernicious anaemia, post gastrectomy states,
nutritional deficiencies, intestinal disorders, and others.
Initially, vitamin B_{12} was assayed microbiologically using

15 either Euglena gracilis or Lactobacillus leichmannii. More
recently, radioisotope dilution (RID) assays for B_{12} have
been utilized. Such radioisotope dilution assay techniques
are well documented in the literature, see for example Lau,
et.al. (1965) "Measurement of Serum B_{12} Levels Using Radio-

20 isotope Dilution and Coated Charcoal," BLOOD, 26, 202, as
modified by Raven et.al. (1968) "The Effect of Cyanide Serum
and Other Factors on the Assay of Vitamin B_{12} by Radio-Isotope
Method Using $^{57}Co-B_{12}$, Intrinsic Factor and Coated Charcoal,"
GUYS HOSPITAL REPORTS, 117, 89; and (1969) "Improved Method

25 for Measuring Vitamin B_{12} in Serum Using Intrinsic Factor,
$^{57}Co-B_{12}$ and Coated Charcoal," JOURNAL OF CLINICAL PATHOLOGY,
22, 205.

Such prior art radioisotope dilution assay of vitamin B_{12}
generally includes the steps of freeing the endogenous B_{12}

30 from its natural binding protein by boiling at a selected pH
and then adding a measured amount of radioisotope $^{57}Co-B_{12}$
and a limited amount of binding protein. All of the binding
protein will be bound by some form of B_{12} since the amount of
radioisotope B_{12} added is, by itself, sufficient to bind the

35 small amount of protein. As both the natural B_{12} and the
radioactive B_{12} compete to bind with the protein, the degree
to which the radioactive count of the protein bound B_{12} was

inhibited was thought to be indicative of the amount of
natural B_{12} present in the sample undergoing testing.

   More specifically, in the technique of Lau et.al. as
modified by Raven et.al., serum B_{12} is separated from
5 binding protein in the plasma sample by boiling with 0.25N
HCl. Radioisotope B_{12} is added to the reaction mixture and
the B_{12} mixture is reacted with protein, normally in the
form of a commercially available binder. Then the free or
unbound B_{12} is separated from the protein bound B_{12} by
10 protein-coated charcoal and the radioactivity of the super-
natant liquid containing the mixture of bound radioactive
B_{12} and bound non-radioactive B_{12} counted for radioactivity.
The serum B_{12} concentration is then calculated from the
count, often by comparison with a standard chart. Almost as
15 soon as this technique began to be utilized it was recog-
nized that the vitamin B_{12} measurements it provided were
usually inconsistent with the results obtained by other meas-
uring techniques for B_{12}, such as the microbiological assays.
Most often, the vitamin B_{12} assay obtained by radioisotope
20 dilution techniques have been found to be high. Many theories
have been advanced to explain the cause of the high vitamin
B_{12} readings. However, it is believed that nowhere in the
prior art is it recognized that there are substances in
mammalian blood and tissue which react with certain non-
25 specific protein binders in the radioisotope dilution assay
technique to provide an analysis of vitamin B_{12} which is
apparently higher than the amount of B_{12} actually in the
sample. Additionally, it is believed that nowhere in the
prior art is it recognized that most common and commercial
30 RID assay protein binders are not specific to vitamin B_{12},
but that they are also capable of binding with the hereto-
fore unknown B_{12} analogues and thus provide erroneous B_{12}
assays.

BRIEF DESCRIPTION OF THE INVENTION

35   As has already been indicated, in the standard radio-
isotope binding assay for vitamin B_{12}, a known amount of
radioactive vitamin B_{12} is mixed with a prepared to-be-
tested sample. Then, a known, but extremely limited, amount

of protein which is capable of binding with both the natural
and radioactive vitamin B_{12} is added to the mixture. Then,
utilizing well known techniques, the radioactivity of the
bound sample is compared, for example, with a standard curve
5 to determine the amount of natural vitamin B_{12} present in
the tested sample. Such standard curves are initially estab-
lished for use in RID assay, for example, by measuring the
amount of bound radioactive B_{12} in the presence of the same
type and amount of protein binder, but with several differ-
10 ent amounts of known non-radioactive B_{12}.

It has now been discovered, for what is believed to be
the first time, that mammalian blood and tissue contain
materials other than vitamin B_{12} which couple with certain
binding proteins which are commonly used in RID assays. For
15 purposes of this specification and claims the non-vitamin
B_{12} materials which are capable of binding with such proteins
will be herein referred to as "vitamin B_{12} analogues,"
"B_{12} analogues" or simply as "analogues." They are referred
to as analogues, not due to their chemical structure, which
20 is not known with certainty, nor in the commonly accepted
chemical sense of the word "analogue." Rather they are
referred to as analogues due to their reactivity with the
binding proteins commonly used in RID assays. As will be
shown in more detail, hereinafter, there are other similar-
25 ities which have been discovered between vitamin B_{12} and
the newly discovered analogues which are present in mammal-
ian blood and tissue.

After the presence of B_{12} analogues was discovered it
was then determined that protein binders commonly present
30 in RID assays were: (1) Non-specific in binding to only
vitamin B_{12}; and (2) reactive in binding with both vitamin
B_{12} and B_{12} analogues; and (3) capable of reacting with both
B_{12} and B_{12} analogues independent of pH. These are most
commonly R proteins. Additionally, it has been determined
35 that other protein binders, are: (1) Very specific in
their reactivity substantially only with vitamin B_{12};
(2) substantially non-reactive with the B_{12} analogues; and
(3) non-reactive with either vitamin B_{12} or B_{12} analogues

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in highly acid environments. These are most commonly
proteins in the form of pure human intrinsic factor (IF),
hog IF, rabbit IF, other IFs and vitamin B_{12} specific
binders.

5   In the past the problem has been that RID binders in-
clude substantial amounts of protein which is not specific
to vitamin B_{12}. Therefore, the radioisotope dilution
assay utilizing that binder on samples which contain B_{12}
analogues will produce a measurement which indicates a
10 greater amount of B_{12} present in the plasma than exists in
fact. As will be shown in more detail hereinafter, commer-
cially available protein binders, which have heretofore been
labeled as containing intrinsic factor, in fact include
only about 10% to about 30% intrinsic factor protein, while
15 the balance of the protein in the binder is of a non-
specific type, such as R protein. Thus, the protein mater-
ials in the commercial protein binders are capable of
indiscriminate reaction with the heretofore unrecognized
vitamin B_{12} analogue materials in mammalian blood and tissue.
20 These extraneous reactions give RID analyses having the
appearance of apparently higher vitamin B_{12} content than the
samples in fact contain. This is due to the fact that when
the binder includes protein which is non-specific to vitamin
B_{12} and which is capable of reacting with both vitamin B_{12}
25 and B_{12} analogues, then the use of this protein in the
radiobinding assay measures both the vitamin B_{12} and the
vitamin B_{12} analogues which are present in the sample. How-
ever, in accordance with the present invention, when the
proteins which are utilized are substantially specific to
30 vitamin B_{12}, such as substantially pure intrinsic factor,
then in the RID assay one binds and measures substantially
only the vitamin B_{12} in the sample, without the measurement
of extraneous B_{12} analogues. This provides a more accurate
vitamin B_{12} RID assay.
35   Based on these discoveries it is proposed that in the
practice of RID assay only protein which is specific in its
reaction to vitamin B_{12} be utilized. Alternatively, it
is proposed that mixtures of vitamin B_{12} specific and

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non-specific binding proteins be treated, for example, with
an excess of material which will bind or inactivate only the
non-specific binding proteins, such as vitamin B_{12} analogues,
prior to its use in RID assays, so that the non-specific

5   protein will be substantially unavailable for reaction with
any vitamin B_{12} or analogues in a sample when the RID assay
is conducted. In yet another modification of the present
invention, crude binder, including non-specific binding pro-
teins, is subjected to proteolytic enzyme treatment prior

10 to utilization as a vitamin B_{12} binder in RID assays. Such
proteolytic enzyme treatment destroys the binding ability
of the non-specific proteins without destroying the binding
ability of the proteins which are specific to vitamin B_{12}.

Utilizing the techniques of the present invention,

15 the B_{12} analogues can by assayed by analyzing the amount of
vitamin B_{12} present utilizing, for example, a vitamin B_{12}
specific binder, then assaying the sample utilizing a non-
specific binder and determining the difference between the
two assays as a measure of the amount of vitamin B_{12} ana-

20 logues present.

These and other techniques are readily determined, once,
as taught for the first time by the present invention, the
presence of B_{12} analogues in mammalian blood and tissue is
recognized.

25 The foregoing and other objects, features and advantages
of the invention will be apparent from the following more
particular description of preferred embodiments of the
invention.

<u>DETAILED DESCRIPTION OF THE INVENTION</u>

30 In the following examples and tables certain chemical
components were utilized. For ease of communication they
have been given shortened names in the text. The con-
cordance between the "component" names and their actual
compositions is as follows:

35 <u>Components</u>   <u>Actual Composition</u>
    A.   Buffer   1.0M Tris (hydroxymethyl)
                                        aminomethane-HCl pH 10.0

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B. Albumin   Bovine serum albumin, 2 mg
                           per ml in H_2O

C. Salt   0.15M NaCl

D. Boiled buffer   (1 part) 0.5M sodium acetate-
5   HCl pH 4.5
                           (1 part) 0.01M KPO_4 pH 7.5,
                           0.15M NaCl
                           (2 parts) 50 µg per ml KCN
                           in 0.15M NaCl

10   The complete solution is heated
                           of 45 min. at 100°C.

E. Standard   Solution D containing 100 pg
   (100 pg/ml B_{12})   per ml vitamin B_{12}. The so-
                           lution is heated for 45 min.

15   at 100°C after the vitamin B_{12}
                           is added. The concentration
                           of vitamin B_{12} in the stock
                           solution used to make component
                           E is determined by its light

20   absorbance at 278, 361 and 550
                           nm.

F. Standard   Same as component E except that
   (1000 pg/ml B_{12})   the vitamin B_{12} concentration
                           is pg/ml.

25 G. (^{57}Co) B_{12} 1000 pg per ml of (^{57}Co) B_{12},
                           (150-300 µCi/µg), in H_2O.

    H. Binder   Present in 0.01M Tris-HCl pH
                           8.2, containing 0.15M NaCl and
                           50 µg per ml bovine serum albumin.

30   Binders are diluted in this
                           solution to reach a concentra-
                           tion of 700 pg per ml of vitamin
                           B_{12} binding ability.
                           Individual binders are as follows:

35   1) Human intrinsic factor
                               (Human IF) -
                               Human gastric juice contain-
                               ing more than 95% intrinsic

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factor based on assays employing inhibition of vitamin B_{12} binding with anti-intrinsic factor antibodies (95% inhibition) and cobinamide (5% inhibition).

2) Human R protein (Human R)-Human saliva containing more than 95% R protein based on assays employing inhibition of vitamin B_{12} binding with cobinamide (95% inhibition) and anti-intrinsic factor antibodies (5% inhibition)

3) Hog intrinsic factor (Hog IF) - This protein was purified from "Hog intrinsic factor concentrate" by affinity chromatography on vitamin B_{12}-Sepharose employing gradient elution with guanidine-HCl followed by gel filtration. The final preparation contained more than 95% intrinsic factor based on assays employing inhibition of vitamin B_{12} binding with anti-intrinsic factor antibodies (95% inhibition) and cobinamide (5% inhibition).

4) Hog R protein (Hog R) (Also designated in the scientific literature as Hog non-intrinsic factor concentrate)

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This protein was purified
from "Hog intrinsic factor
concentrate" as described
above in 3). The final
preparation contained more
than 95% R protein based
on assays employing inhi-
bition of vitamin B_{12} bind-
ing with anti-intrinsic
factor antibodies (5%
inhibition) and cobinamide
(95% inhibition).

5) Rabbit intrinsic factor
    (Rabbit IF) - An extract
    of rabbit gastric mucosa
    containing more than 95%
    intrinsic factor based on
    assays employing inhibition
    of vitamin B_{12} binding with
    anti-intrinsic factor anti-
    bodies (95% inhibition)
    and cobinamide (5% inhi-
    bition).

6) Hog intrinsic factor concen-
    trate (Hog IFC) - A crude
    extract of hog pyloric mucosa.
    It contained 25% Hog IF and
    75% Hog R based on assays
    employing inhibition of
    vitamin B_{12} binding with
    anti-intrinsic factor
    antibodies (25% inhibition)
    and cobinamide (75% inhi-
    bition).

7) Hog IFC + Cobinamide - Hog
    IFC containing the vitamin
    B_{12} analogue cobinamide

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([CN, OH] Cbl) in a molar amount equal to 100 times the total vitamin B_{12} binding ability, i.e. a 100 fold excess of cobinamide.

8) Hog IFC + CN-Cbl [bde-OH] – The same as item 7) above except that the analogue added is CN-Cbl [bde-OH] and is present in a 1000 fold molar excess.

9) Hog IFC + [3,5,6-Me_3BZA] (CN,OH)Cba – The same as item 7) above except that the analogue added is [3,5,6-Me_3BZA] (CN, OH)Cba.

10) Digested Hog IFC – Hog IFC incubated with bovine pancreatic trypsin (2 mg per ml) and bovine pancreatic chymotrypsin (2 mg per ml) for 60 min. at 37°C.

I. Charcoal

A solution containing 25 mg per ml neutral charcoal (Norit) and 5 mg per ml bovine serum albumin in H_2O.

J. unknown sample

Samples containing unbound vitamin B_{12} are diluted in solution D (see above). Samples containing bound vitamin B_{12}, such as serum, are prepared as follows:

(1 part) sample

(1 part) 0.5M sodium acetate–HCl pH 4.5

(2 parts) 50 µg per ml KCN in 0.15M NaCl

10

The complete mixture is heated
at 100°C for 45 min. followed
by centrifugation at 5000x g at
4°C for 20 min. The superna-
5   tant is removed and used for
assay.

Each of the RID assays referred to herein utilized the
components referred to above. The method and order of
utilizing the components is that set forth in Table 1. That
10 is, components A, B, C, etc. or the buffer, albumin, and
salt, respectively, etc. were added in the order, from left
to right, shown in Table 1. After the addition of $^{57}Co-B_{12}$
the components are mixed thoroughly to mix both the naturally
occurring B_{12} and the radioisotope B_{12} to make them compete
15 and equally available to react with the binder. After the
addition of the binder, H, the components were again mixed
thoroughly, and then incubated for 45 minutes at about 37°C.
Charcoal was then added to the incubated mixture and the com-
ponents again mixed thoroughly and incubated for another
20 5 minutes at room temperature. This was followed by cen-
trifuging at 2000 x g at 4°C for 30 minutes. Then 1000 µl
of the resulting supernatant liquid is pipetetted from the
sample and a determination of the amount of $^{57}Co-B_{12}$ present
is made. The amount of $^{57}Co-B_{12}$ is indicative of the amount
25 of natural B_{12} in the tested sample, with lesser amounts of
$^{57}Co-B_{12}$ being indicative of greater amounts of natural
vitamin B_{12} in the sample.

Calculations of vitamin B_{12}, utilizing the datea obtained
in the foregoing manner, is made as follows:
30

Calculation of data from radiobinding assay for plasma
vitamin B_{12} assay as outlined in Table I
1) The values in tubes 3 and 4, the "blank" tubes without
    binder are averaged and subtracted from all other tubes
35   starting with tube 5.
2) The background radiation is subtracted from tubes 1 and
    2 and these values are averaged.
3) Tubes 5 and 6 are averaged. This value should be at

Table I

Flow Sheet for Radiobinding Assay for Vitamin B12

Tube #	A Buffer (ul)	B Albumin (ul)	C Salt (ul)	D Boiled Buffer (ul)	E Standard 100pg/ml B12 (ul)	F Standard 1000pg/ml B12 (ul)	J Unknown Sample (ul)	G [57Co] B12 (ul)	H Binder (ul)	I Charcoal (ul)
1,2	225	50	875	800	-	-	-	50	-	-
3,4	225	50	375	800	-	-	-	50	-	500
5,6	225	50	325	800	-	-	-	50	50	500
7	225	50	325	720	80	-	-	50	50	500
8	225	50	325	640	160	-	-	50	50	500
9	225	50	325	560	240	-	-	50	50	500
10	225	50	325	300	500	-	-	50	50	500
11	225	50	325	720	-	80	-	50	50	500
12	225	50	325	640	-	160	-	50	50	500
13	225	50	325	450	-	350	-	50	50	500
14	225	50	325	-	-	800	-	50	50	500
Unknown Sample	225	50	325	-	-	-	800	50	50	500

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least 15% below the average value for tubes 1 and 2 to
insure that all of the binder is saturated in the
presence of (^{57}Co) B_{12} alone.

4) Values for each tube beginning with tube 7 are divided
   by the average of tubes 5 and 6 to give values for "%
   trace binding."

5) Percent trace binding for tubes 7-14 are used to
   obtain a standard curve. We plot % trace binding on
   the ordinate of logit-log paper versus pg vitamin B_{12}
   on the log scale.

6) The amount of vitamin B_{12} in unknown samples is
   determined by interpolation from the standard curve or
   data of % trace binding versus pg vitamin B_{12}.

7) The standard curves for all of the various binders used
   are virtually indistinguishable and vary little from
   day to day. Nevertheless, a complete standard curve is
   always obtained for every binder with each set of assays.
   Representative data obtained with the assays are pres-
   ent in Table II.

Evidence as to the Origin and Existence of Vitamin B_{12}
Analogues in Mammalian Blood and Tissue

Once the problem of the prior art is recognized, that
is, that there are vitamin B_{12} analogues present in mammal-
ian blood and tissue, it becomes a relatively simple matter
to prove the existence and chemistry of such analogues. It
is also appropriate to prove that the various steps of the
RID assay do not cause the B_{12} analogues to be formed, for
example, from vitamin B_{12}.

In one instance this has been most convincingly shown
by obtaining pure crystalline vitamin B_{12}, subjecting var-
ious known concentrations of it to the same conditions used
to extract endogenous vitamin B_{12} from blood and tissue sam-
ples (boiling for 45 minutes in the same extraction solution)
and then analyzing them by RID assay using several binding
proteins, for example, in the form of human IF, hog IF,
human R, hog R and hog IFC on different portions of the
same extracted vitamin B_{12} samples.

Table II

Standard Curves obtained with the radiobinding assay for vitamin B12 using various binders

Vitamin B12 added(a) (pg)	% trace binding observed with various binders(b)				
	Human IF	Human R	Hog IF	Hog R	Hog IFC
0	(100)	(100)	(100)	(100)	(100)
8	90	93	94	89	89
16	85	84	83	83	80
24	79	78	74	76	76
50	64	60	58	56	59
80	45	44	44	43	43
160	30	28	28	27	28
350	15	14	15	14	14
800	6	6	7	6	7

(a) The vitamin B12 was boiled for 45 min. at 100°C in the same solution used to extract endogenous vitamin B12 from human plasma.

(b) Assays were performed on different days.

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Referring to Table II, it will be seen that when various known amounts of pure vitamin B_{12}, ranging from about 8 pg to about 800 pg were tested with various protein binders, that in each instance, the percent of

5   radioactive trace binding, or more accurately, the inhibition of $^{57}Co-B_{12}$ binding, observed was substantially the same for each binder. It is thus seen, that regardless of which protein binder is utilized, the percent binding, i.e. inhibition of the $(^{57}Co)-B_{12}$ is substan-

10   tially the same. This is indicative of the fact that during preparation for RID assay the pure vitamin B_{12} was not converted to analogues of the type which have now been observed in mammalian blood and tissue. It is also indicative of the fact that in the absence of interferring masking

15   components in the samples, such as B_{12} analogues, any of the binding proteins can be utilized to provide substantially equally acurate RID assays of vitamin B_{12}. Furthermore, the data in Table II should be suitable as a standard in the determination of vitamin B_{12} by the same

20   RID assay.

By comparison, when endogenous vitamin B_{12} was extracted from serum from 74 normal blood donors (37 women, 37 men, ages 17-61) and tested utilizing the same binding proteins with the exception of hog IFC which was not used, the results

25   were quite different. In every case in which serum from normal donors was tested greater inhibition of $^{57}Co-B_{12}$, and therefore greater apparent vitamin B_{12}, was observed with assays employing, as the binder, human R or hog R than was observed with assays employing human IF or hog IF.

30   The data on the 74 normal donors is included in Table III. Other data concerning patients with diagnosed vitamin B_{12} deficiencies are present, and comparisons between the normal donors and patients have also been made on Table III, and will be discussed in more detail hereinafter. Referring

35   to Table III it is seen that the mean endogenous vitamin B_{12} RID assay levels, in terms of pg of vitamin B_{12} per ml of serum, are 548 and 542 for human R and hog R,

Table III

Distribution of serum vitamin B_{12} values as measured with various binders for 74 normal subjects and 21 patients with clinical evidence of vitamin B_{12} deficiency
All assays were performed at pH 9.0

Serum Vitamin B_{12} (pg/ml)	Human R		Hog R		Human IF		Hog IF		Human R minus Human IF		Hog R minus Hog IF	
	Normals	Patients	Normals	Patients	Normals	Patients	Normals	Patients	Normals	Patients	Normals	Patients
0-24					8		11					
25-49					6		5					
50-74					5		3			1	2	1
75-99		1		1	2		2		2		3	
100-124		2		1					2	3	9	3
125-149		2		5	4		1		7	3	5	3
150-174		3		1	5		3		6	5	9	5
175-199		4		3	2		3		6	3	8	3
200-224	1	1		2	5		4		8	2	11	2
225-249		3	1	1	6		4		2	3	6	3
250-274				3	10		9		10		4	
275-299		1	2	1	4		3		5		1	
300-324	1	2	4	2	12		4		6		3	
325-349	3	1	2	1	5		6		4	1	5	1
350-374	5	1	1				10		4		1	
375-399	3	2			3		4		4		2	
400-424	5	3			4		5		5		1	
425-449	3	4			3		2		2			
450-474	4	6			2		3				1	
475-499	5	5					3				1	
500-524	7	4			1		3					
525-549	3	4			1							
550-574	5	3			2		1				1	
575-599	1	2			1				1			
600-624	2	4			1		3				1	
625-649	5	4										
675-699	2	3										
700-724	3	6					1					
725-749	2											
750-774	3	1					1					
775-799		1			1							
800-824		2										
825-849												
850-874	1	1										
875-899	1	2					1					
900-924	1	1										
925-969	2	1										
950-974	1											
975-999	1											
1000-1024												
1025-1049												
1050-1074	1											
1075-1099												
1100-1124												
1125-1149		1										
1150-1174												
1175-1199												
1200-1224												
1225-1249	1											
range of serum B_{12}	220-1230	85-355	245-1135	84-342	130-785	0-78	135-880	0-86	70-575	56-337	50-605	72-332
mean of serum B_{12} (a)	348		542		298	36	361	33	254	169	212	171
mean ± 2 Std. Dev.	282-1040		276-1065		136-656		157-717		70-661		58-531	

(a) Based on values obtained with log vitamin B_{12}. Log values were used since these gave a normal curve while the curve using untransformed values were skewed to the right.

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respectively, but only 298 and 3.61 for human IF and hog
IF, respectively. This demonstrates that something is
present in extracts of normal human serum which inhibits
the vitamin ^{57}Co-B_{12} binding ability of human R and hog

5   R to a greater extent than it inhibits the binding ability
of human IF and hog IF. Under current RID assay tech-
niuqes the greater inhibition which is found using human
R and hog R is analyzed to indicate a higher vitamin B_{12}
content. It is those substances, which have now been found

10 to be present in human blood serum and which preferentially
inhibit ^{57}Co-B_{12} binding of human R and hog R, which have
been herein denominated as "vitamin B_{12} analogues."

Chemical Nature and Properties of Vitamin B_{12} Analogues

    The vitamin B_{12} analogues, which are herein for the

15 first time identified as being present in mammalian blood
and tissue, have been isolated by paper chromatography and
compared with pure vitamin B_{12}. Vitamin B_{12} and the so-
called "vitamin B_{12} analogues" were found to have the
following properties in common: (1) Both were adsorbed

20 to charcoal and remained adsorbed when the charcoal was
washed with 5% phenol; (2) Both were eluted from charcoal
when the charcoal was washed with 67% acetone; (3) Both
were extracted from aqueous solution into phenol and re-
mained in the phenol phase even when the phenol was washed

25 repeatedly with water; (4) Both passed into the aqueous
phase when the phenol layer was dissolved in an excess of
diethyl ether; (5) Both eluted with similar apparent
molecular weights (approximately 1356) during gel filtra-
tion on columns of Bio-Rad P-4 polyacrylamide; (6) Both

30 were adsorbed to a column of Sepharose-2B agarose that
contained covalently bound hog R protein and both remained
bound when the column was washed with 0.1M glycine-NaOH
pH 10.0, 1.0M NaCl, and both were eluted from the Sepharose
with either 85% phenol or 60% pyridine. Because of these

35 similarities the newly discovered material is seen to be
similar to vitamin B_{12} and is thus referred to as vitamin
B_{12} analogue.

17

The chemical nature and structure of the newly
discovered vitamin B_{12} analogues which are now found to be
present in mammalian blood and tissue is not known. An
effort was made to compare them with chemically true forms

5   of vitamin B_{12}, sometimes referred to in the literature as
analogues of vitamin B_{12}, namely CN-B_{12}, OH-B_{12}, adenosyl-
B_{12} and CH$_3$-B_{12}, already known to be present in serum and
tissues. This was done by adding 500 pg of each of these
four known forms of vitamin B_{12} to four different portions

10 of the same human serum, in the dark. Prior to the
additions the serum contained 250 pg and 450 pg of vitamin
B_{12} as assayed by RID using human IF and human R, respec-
tively, thus exhibiting a difference of 200 pg. After ad-
dition of the materials to the serum, each was allowed to

15 incubate in the dark for 15 minutes to allow binding of the
added known forms of vitamin B_{12} to the binding proteins
normally present in the serum. Then the serum, with the
added forms of vitamin B_{12} was extracted utilizing standard
conditions and the apparent amount of vitamin B_{12} assayed

20 by RID utilizing both human R protein and human IF protein.
Both the human R and human IF assays showed an increase
in the apparent amount of vitamin B_{12} of about 500 pg.
However, the original difference observed between the values
obtained with the human R protein and the human IF protein,

25 i.e. 200 pg, did not change. If any of the added known
forms of vitamin B_{12} in the human serum had been converted
to the newly discovered analogues, then the assays would
have shown an increase in the difference. This provides
evidence that the newly discovered vitamin B_{12} analogues

30 were not formed from any of the known endogenous forms of
native viatime B_{12} during the extraction procedure.

Isolation of Vitamin B_{12} Analogue

The materials which are herein designated as "vitamin
B_{12} analogues" and which have been found to preferentially

35 inhibit R proteins in the vitamin B_{12} assays were substan-
tially separated from endogenous vitamin B_{12} by the follow-
ing purification scheme. A trace amount of 150 pg ^{57}Co-B_{12},
was added to 1800 ml of freshly collected normal human

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plasma. The added $^{57}Co-B_{12}$ was sufficiently small that
it did not interfere with subsequent RID assays. After
incubating at room temperature for 30 minutes the vitamin
B_{12} was extracted and assayed under standard conditions.

5 When human IF binder was utilized in the RID assay the extract
was found to contain 1050 ng of vitamin B_{12}, but when human
R protein was utilized as the binder it appeared to con-
tain 2030 ng of vitamin B_{12}, almost twice as much vitamin
B_{12}. The extract was then passed through a column of

10 Sepharose containing covalently bound hog R protein. The
column retained greater than 99% of the $^{57}Co-B_{12}$ as well as
the endogenous vitamin B_{12} as assayed by RID with human IF
or human R protein. After the column was washed with a
variety of buffers and water the material was eluted with

15 60% pyridine, taken to dryness under vacuum, dissolved in
water, and adsorbed onto charcoal. The charcoal was
washed with 5% phenol followed by water and the mixture of
vitamin B_{12}, $^{57}Co-B_{12}$ and analogue B_{12} was eluted from the
charcoal with 67% acetone. The material was again taken

20 to dryness under vacuum, dissolved in water, and then sep-
arated utilizing 19 inch long Whatman 3MM paper for paper
chromatography and a solvent system consisting of 800 ml
sec-butanol, 8 ml glacial acetic acid, 6 mmol HCN and 400
ml water. The chromatography was performed in the descend-

25 ing manner for 30 hours at room temperature in an environ-
ment that inhibited evaporation of the solvent. The paper
chromatogram was allowed to dry in a fune oven and divided
into 38 one-half inch fractions and numbered, with fraction
1 starting at the point of application and number 38 being

30 at the lowest point on the chromatogram. Each one-half
inch fraction was then incubated with 5 ml of water at 4°C
for twelve hours to elute the vitamin B_{12}, $^{57}Co-B_{12}$ and
B_{12} analogues. The water was then removed and taken to dry-
ness under vacuum. Each dried fraction was then dissolved

35 in 2.5ml of water and assayed for $^{57}Co-B_{12}$ and for vitamin
B_{12} using a variety of binding proteins. The final
recovery of $^{57}Co-B_{12}$ was 64%. The apparent recoveries
of vitamin B_{12} were 75% when using human IF in the assay

19

and 66% when using human R in the assay. The results of
the assays employing the 38 fractions obtained by paper
chromatography are presented in Table IVA, IVB, and IVC.
Similar data concerning paper chromatography of $^{57}Co-B_{12}$

5 and pure vitamin B_{12}, for reference as a control, are
presented in Table IVD. The data in these several chroma-
tography tables summarized for convenience in Table IVE
reveals that the behavior of $^{57}Co-B_{12}$ that was extracted
from human plasma did not change its chromatographic behav-

10 ior, and thus was not altered during the standard extraction
procedure or any of the purification steps. In a similar
manner it is postulated that true vitamin B_{12} is not
altered in any of the purification or process steps of the
assay.

15   Referring to the control chromatogram of Table IVD, it
is seen that the several RID assays of pure vitamin B_{12}
performed variously with human R protein, human IF protein
and hog IFC gave substantially a single symmetrical peak
of activity. In each instance greater than 95% of the

20 vitamin B_{12} was found to be present in fraction 14 through
16. Similar results, as shown in Table IVB, were obtained
from the paper chromatogram of the plasma extract when the
binding protein was human IF, hog IF and rabbit IF. These
data are an indication that these three IF binding proteins

25 are substantially specific in their binding ability to vita-
min B_{12}, and substantially non-reactive with vitamin B_{12}
analogues present in plasma.

       Efforts were made to modify hog IFC, which is a commonly
used binder in RID assays and which has been found to con-

30 tain as much as 90% hog R protein and as little as 10% hog
IF, by removing or inactivating the hog R. In several in-
stances the hog IFC was incubated with an excess amount of
three chemically synthesized vitamin B_{12} analogues before
it was utilized in the RID assay. Referring to Table IVC,

35 it is seen that after this modification the chromatogram
results obtained utilizing the modified hog IFC closely
resemble the results obtained with substantially pure hog
IF. It is therefore seen, that in the practice of the

Table IVA

[57Co] B12 and plasma B12 assayed after elution from paper chromatography. All assays were performed at pH 9.0 except where indicated.

Fraction #	[57Co] B12 (cpm)	[57Co] B12 (%)	B12 assayed with various binders									
			Human R (ng)	Human R (%)	pH 1.8 Human R (ng)	pH 1.8 Human R (%)	Hog R (ng)	Hog R (%)	pH 1.8 Hog R (ng)	pH 1.8 Hog R (%)	Hog IFC (ng)	Hog IFC (%)
1			2				1		1		1	
2												
3												
4			1		1		1		2		1	
5			2				2		3		2	
6			3		3		2		4		2	
7			4		4		4		5		3	
8			4		3		4		5		3	
9			7	1	6		6	1	8	1	5	1
10	300	1	24	2	29	2	26	2	29	2	16	2
11	500	2	42	3	54	4	36	3	49	3	26	3
12	300	1	25	2	34	2	26	2	38	2	19	2
13	200	1	31	2	44	3	30	3	55	4	22	2
14	4600	20	209	16	235	16	176	16	240	16	180	17
15	14000	61	562	42	615	43	461	41	576	37	474	46
16	2100	9	103	8	122	9	84	7	122	8	91	9
17	300	1	85	6	118	8	85	8	128	8	53	5
18	300	1	68	5	68	5	68	6	100	6	45	4
19	200	1	23	2	17	1	17	2	27	2	15	1
20			45	3	39	3	39	3	60	4	24	2
21			42	3	25	2	25	2	52	3	21	2
22			8	1	5		7	1	8	1	5	1
23			8	1	4		5		8	1	4	
24			7	1	4		5		7		3	
25			9	1	1		6	1	3		4	1
26			16	1	1		10	1	4		5	
27			5		1		3		2		2	
28			2				1		1		1	
29			4				2		1		2	
30			1				1				1	
31			1									
32-38												

Table IIB

Assay of [57Co] B12 and plasma B12 after elution from paper chromatography.
All assays were performed at pH 9.0.

Fraction #	[57Co] B12 (cpm)	(%)	B12 assayed with various binders					
			Human IF (ng)	(%)	Hog IF (ng)	(%)	Rabbit IF (ng)	(%)
1			1				1	
2								
3								
4								
5					1			
6					1		1	
7					1		1	
8					1		1	
9			1		1		2	
10	300	1	4	1	5	1	8	1
11	500	2	7	1	10	1	16	2
12	300	1	5	1	7	1	10	1
13	200	1	14	2	10	1	13	1
14	4600	20	169	21	169	22	170	19
15	1400	61	488	61	450	57	480	55
16	2100	9	73	9	72	9	87	10
17	300	1	17	2	25	3	42	5
18	300	1	11	1	15	2	25	3
19	200	1	3		5	1	5	1
20			5	1	7	1	8	1
21			2		4		4	
22-38								

Table

Assay of [57Co] B12 and plasma B12 after elution from paper chromatography. All assays were performed at pH 9.0.

B12 assayed with various binders

Fraction #	[57Co] B12 (cpm)	[57Co] B12 (%)	Hog IFC (ng)	Hog IFC (%)	Hog IFC + Cobinamide (ng)	Hog IFC + Cobinamide (%)	Hog IFC + CN-Cbl[bde-OH] (ng)	Hog IFC + CN-Cbl[bde-OH] (%)	Hog IFC + [3,5,6-Me3BZA](CN, OH)Cba (ng)	Hog IFC + [3,5,6-Me3BZA](CN, OH)Cba (%)	Hog IFC Digested (ng)	Hog IFC Digested (%)
1			1									
2												
3											1	
4			1								1	
5			2				1				1	
6			2				1		1		1	
7			3				1		1		2	
8			3				1		1		2	
9			5	1	1		1		2		3	
10	300	1	16	2	4		5	1	7	1	9	1
11	500	2	26	3	10	1	10	1	13	2	15	2
12	300	1	19	2	8	1	8	1	9	1	11	1
13	200	1	22	2	9	1	10	1	12	2	14	2
14	4600	20	180	17	174	21	170	22	148	20	153	19
15	14000	61	474	46	474	58	435	55	396	53	402	50
16	2100	9	91	9	83	10	81	10	80	11	84	10
17	300	1	53	5	25	3	27	3	34	4	36	4
18	300	1	45	4	16	2	19	2	21	3	27	3
19	200	1	15	1	4		4	1	6	1	9	1
20			24	2	7		7	1	11	1	13	2
21			21	2	3	1	4	1	7	1	9	1
22			5	1					1		2	
23			4						1		1	
24			3								1	
25			4								2	
26			5	1								
27			2									
28			1									
29			2									
30			1									
31-38											1	

Table IID

Assay of [^{57}Co] B$_{12}$ and native B$_{12}$ after elution from paper chromatography. All assays were performed at pH 9.0.

Fraction #	[^{57}Co] B$_{12}$ (cpm)	(%)	B12 assayed with various binders					
			Human R (ng)	(%)	Human IF (ng)	(%)	Hog IFC (ng)	(%)
1								
2								
3								
4								
5								
6								
7								
8								
9								
10	200	1	1				1	
11	600	2	1		1		2	1
12	400	1	1		1		2	1
13	400	1	4	1	3	1	6	2
14	6800	24	86	25	86	26	90	27
15	15800	56	210	60	198	60	189	56
16	3100	11	40	12	38	12	40	12
17	200	1	2	1	1		3	1
18	200	1	2	1	1		2	1
19	100		1				2	
20-38								

Table IVE

Summary of the data in Tables IVA - IVD involving assays of [57Co] B_{12} and B_{12} after elution from paper chromatography. Assays for B_{12} were performed at pH 9.0 except where indicated.

Sample Chromatographed	Item Assayed	Assay Binder	1-13 (ng)	1-13 (%)	14-16 (ng)	14-16 (%)	17-38 (ng)	17-38 (%)	1-38 (ng)	1-38 (%)
[57Co] B_{12} + B_{12}	[57Co] B_{12}	-	-	5	-	92	-	2	-	100
"	B_{12}	Human R	7	2	336	97	5	1	348	100
"	B_{12}	Human IF	5	1	322	98	2	1	329	100
"	B_{12}	Hog IFC	11	3	319	95	7	2	337	100
[57Co] B_{12} + plasma B_{12}	[57Co] B_{12}	-	-	6	-	91	-	3	-	100
"	B_{12}	Human R	145	11	874	65	324	24	1343	100
"	B_{12}	Human R (pH 1.8)	179	12	972	68	283	20	1434	100
"	B_{12}	Hog R	138	12	721	64	274	24	1133	100
"	B_{12}	Hog R (pH 1.8)	199	13	938	61	402	26	1539	100
"	B_{12}	Hog IFC	100	10	745	72	185	18	1030	100
"	B_{12}	Human IF	31	4	730	91	38	5	799	100
"	B_{12}	Hog IF	37	5	691	88	56	7	784	100
"	B_{12}	Rabbit IF	53	6	737	84	84	10	874	100
"	B_{12}	Hog IFC + Cobinamide	32	4	731	89	54	7	818	100
"	B_{12}	Hog IFC + CN-Cbl[bde-OH]	47	6	624	83	81	11	752	100
"	B_{12}	Hog IFC + [3,5,6-Me3BZA](CN,OH)Cba	38	5	686	87	61	8	785	100
"	B_{12}	Hog IFC - Digested	60	7	639	80	102	13	801	100

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present invention, mixtures of protein including both
vitamin B_{12} specific binding protein and binding protein
which is not specific to vitamin B_{12} can be modified by
the addition of an excess amount of vitamin B_{12} analogue,
5   by which process the analogue binds with the non-specific
protein to render it substantially bound or inactive so
that it is not available to react with vitamin B_{12} or
(^{57}Co) B_{12} present in samples undergoing RID tests. The
amount of vitamin B_{12} analogue to be added to a mixture of
10   specific and non-specific proteins in order to bind or in-
activate the non-specific proteins may vary over a wide
range, depending on both the proteins which are present and
the vitamin B_{12} analogues which are utilized as the binding
or inactivating material. Generally speaking, for the exam-
15   ples shown in Table IVC, cobinamide may be added in an
amount equal to that required for complete binding of the
non-specific protein, up to an amount as much as ten million
times greater than the amount needed to bind the protein,
with the preferred range being about ten to about ten
20   thousand times in excess of that required for complete bind-
ing. CN-Cb (bde-OH), known as CoB-cyano-cobamic a,c,g-
triamide may be utilized in an amount at least about ten
times to about ten million times in excess of the amount
required to bind with the non-specific protein, with an
25   amount of about one hundred to about one hundred thousand
times excess being preferred. The $(3,5,6-Me_3BZA)$
$(CN,OH)-cba$, known as Co $(3,5,6-trimethylbenzimidazole)$
cobamide should also be utilized in amounts from about
one to ten million times in excess of the amount of non-
30   specific protein, with an amount in the range of about ten
to about ten thousand times excess being preferred. Suit-
able amounts of other vitamin B_{12} analogues may be utilized
in a similar manner to bind or inactivate non-specific pro-
teins present in mixtures with specific proteins in order
35   to obtain a preparation of binding protein which will
substantially bind only $^{57}Co-B_{12}$, or the vitamin B_{12}

naturally present in the samples being tested, and thus

give a more accurate quantitative RID assay of vitamin B_{12} in samples undergoing tests.

    Again, referring to Table IVC, data on samples of hog IFC digested with trypsin and chymotrypsin are shown. These

5 and other proteolytic enzymes are specific in their ability to substantially digest R proteins while leaving intrinsic factor proteins unaffected and available as substantially the only protein for binding ^{57}Co-B_{12} and vitamin B_{12} in RID assays. Other enzymes, including, for example, elastase

10 may be utilized for the same purpose. The amount of the enzymes utilized is in the range of about 0.01 to about 100 milligrams per milliliter of protein treated, with a preferred amount being about 0.05 to about 40 milligrams per milliliter of protein. Utilizing this proteolytic enzyme

15 digestion process a protein binder is provided which substantially binds only ^{57}Co-B_{12} and vitamin B_{12} and is not affected by vitamin B_{12} analogues in the samples being tested and which therefore gives a more accurate RID assay than is obtained when utilizing the original mixture of hog

20 IFC proteins which included non-specific proteins which would have been capable of reacting with the newly discovered vitamin B_{12} analogues in samples to give inaccurate assays as to the amount of vitamin B_{12} in test samples.

    Now, referring to Table IVA, when samples from the

25 plasma extract chromatogram were assayed for vitamin B_{12} with human R, hog R and hog IFC, different results were obtained than when those samples were assayed with human IF, hog IF, rabbit IF or hog IFC treated with vitamin B_{12} analogues or hog IFC digested with proteolytic enzymes.

30 In each case where human R, hog R or untreated hog IFC were utilized as the binding protein the tests gave the appearance that more vitamin B_{12} was present in the chromatogram samples, especially in fraction 1 through 13 and 17 through 38. This observation, when taken with the above data, pro-

35 vides strong evidence that normal human plasma contains a number of vitamin B_{12} analogues that compete with ^{57}Co-B_{12}, in significant amounts, for binding to R protein. It also indicates that such activity on the part of the B_{12}

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analogues is substantially absent when the binding protein
utilized in the RID assay is substantially specific to
vitamin B_{12}.

It should also be noted, see Table IVA, that the
5   chromatogram data suggests that the lack of specificity of
human R and hog R is unchanged when RID assays are performed
at acid pH. This indicates that erroneous results will be
obtained for the true vitamin B_{12} content of samples which
contain vitamin B_{12} analogues when RID assays are performed
10 at acid pH.

Using the same techniques and criteria described above
it has been discovered that vitamin B_{12} analogues are not
only present in serum obtained from human blood, but are
also present in mammalian tissues in even higher concen-
15 trations than they are in blood. Vitamin B_{12} analogues
extracted from mammalian tissues have been purified using
the same schemes as described above. When analyzed util-
izing paper chromatography, they exhibited similar mobil-
ities to those of the vitamin B_{12} analogues observed in
20 the samples extracted from blood serum. Since larger
amounts of the vitamin B_{12} analogues are present in tissue,
they can be observed visually as red or orange spots during
paper chromatography. The absorption spectra of vitamin
B_{12} analogues purified from tissue extracts have been de-
25 termined and demonstrate that they are similar to, but dis-
tinct from, the absorption spectrum of true vitamin B_{12}.
These observations provide additional evidence that the
materials in blood serum which preferentially react with
R proteins and not intrinsic factor proteins are in fact
30 varieties of vitamin B_{12} analogues.

The newly discovered vitamin B_{12} analogues also differ
from vitamin B_{12} in terms of their biological activity.
Thus, as shown in Table V, the serum vitamin B_{12} values ob-
tained with _Euglena gracilis_ for eleven patients diagnosed
35 to have vitamin B_{12} deficiency were substantially similar to
the results obtained by RID assay using human IF or hog IF
as the binding protein. It is to be further noted, that
all of the values obtained by either microbiologic assay or

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Table V

Vitamin B_{12} levels in 21 patients with clinical evidence of vitamin B_{12} deficiency

| Patient | Vitamin B12 assayed with various binders | | | | Vitamin B_{12} assayed with Euglena Gracilis |
	Human R (pg/ml)	Hog R (pg/ml)	Human IF (pg/ml)	Hog IF (pg/ml)	(pg/ml)
1	155	138	16	0	43
2	310	295	51	86	52
3	310	255	24	35	46
4	135	132	0	22	40
5	215	250	52	60	25
6	347	342	0	0	56
7	102	120	0	0	35
8	240	242	58	65	79
9	160	125	38	23	0
10	85	84	0	12	23
11	235	255	5	11	0
12	188	190	41	38	
13	178	192	48	38	
14	298	305	53	57	
15	355	310	78	82	
16	178	210	48	22	
17	128	140	31	8	
18	106	190	50	40	
19	163	155	0	0	
20	178	132	25	0	
21	230	215	75	41	
Mean (1-11)	209	203	22	29	36
Mean (1-21)	205	204	33	30	
Normal Range (mean ± 2 std. dev.)	220-1230	245-1135	136-656	157-717	(>130)
Number within normal range	9 (43%)	10 (48%)	0	0	0

by assay using human IF or hog IF were substantially lower
than the values obtained when the RID assay was carried
out utilizing human R or hog R as the binding protein.
This indicates that the vitamin B_{12} analogues which have now

5   been identified in mammalian blood and tissue do not possess
vitamin B_{12} activity of the type which is required to pro-
mote the growth of Euglena gracilis.

Data was obtained on ten additional patients diagnosed
to be vitamin B_{12} deficient and the total of 21 patients

10 with vitamin B_{12} deficiency are shown in Table V. In each
of the 21 patients the vitamin B_{12} values found when the
RID assay was carried out utilizing human IF or hog IF
were below the range of vitamin B_{12} values found in a control
group of 74 normal subjects. However, when the RID assay

15 was carried out utilizing human R or hog R only about half
of the 21 vitamin B_{12} deficient patients were found to
assay below the range of normal subjects for vitamin B_{12}.
This indicates that where the newly found vitamin B_{12}
analogues are present in the samples being tested, and the

20 binding protein is not specific to vitamin B_{12}, the result-
ing assays may suggest that a truly vitamin B_{12} deficient
patient is not within the deficient range. This may lead
to delay of treatment of that patient for vitamin B_{12}
deficiency. It also indicates that the vitamin B_{12} ana-

25 logues that have now been discovered lack the therapeutic
or beneficial activity of vitamin B_{12} in the sense of being
unable to prevent the hematologic and/or neurologic
abnormalities associated with vitamin B_{12} deficiency.

30 There are many commercial RID assay type kits available
for the assay of vitamin B_{12} in clinical laboratories.
Table VI sets forth an analysis of several such kits, and
a comparison of the types of protein found in those kits
with hog IF, hog R and hog IFC. By reference to Table VI,

35 it is seen that the commercial kits appear to have only
about 13% to about 35% intrinsic factor and from about 60%
to about 85% R.protein. It is therefore suspected, that
the use of these kits will give substantially erroneous
assays of the amount of vitamin B_{12} present in a sample

Table VI

Analysis of vitamin B12 binders and assay pH used in commercial kits sold for the assay of vitamin B12 in clinical laboratories -

Source of Binder	Vitamin B12 Binding Protein		Assay pH of Kit(d)
	Intrinsic factor(a) (%)	R protein(b) (%)	
Hog IF	97	0	-
Hog R	0	98	-
Hog IFC	25	75	-
Diagnostic Products Corp. Kit	35	60	1.7
New England Nuclear Kit	20	82	4.1
Bio-Rad Laboratories Kit	30	71	1.9
Medvak Diagnostic Products Kit	13	85	1.8
Schwarz/Mann Kit	34	67	9.1
Pharmacia Diagnostics Kit	1 (33)c	67	4.1

(a) Inhibition of [57Co] B12 binding observed with anti-IF antibodies at pH 7.5.

(b) Inhibition of [57Co] B12 binding observed with a 100 fold molar excess of cobinamide at pH 7.5.

(c) In this kit the binder is covalently attached to an insoluble matrix and because of steric factors it may not be accesible to anti-intrinsic factor antibodies. Thus the value for % intrinsic factor may be as high as 33%.

(d) Refers to the pH measured after the addition of all of the components of each individual kit. R protein retains its full B12 binding ability over the range of pH 1.7-9.1 but intrinsic factor loses 10% and 99% of its B12 binding ability at pH 4.1 and 1.9, respectively. Thus kits that use pH 4.1 have slightly less % intrinsic factor than is indicated in the table while those that use pH 1.7-1.9 are employing essentially no intrinsic factor.

when the sample also includes vitamin B_{12} analogues, such
as those newly discovered to exist in mammalian blood and
tissues. It has also been determined that the effective-
ness of intrinsic factor to bind vitamin B_{12} is somewhat
5 pH dependent, with intrinsic factor losing about 10%
of its binding ability at a pH of about 4.1 and losing about
99% of its binding ability at a pH of about 1.9. Thus, to
the extent that the commercial kits use a pH of about 4.1
during binding they would have about 10% less intrinsic
10 factor than shown in Table VI. Those kits having a pH
during binding of about 1.7 to about 1.9 would obtain
substantially no binding from intrinsic factor.

   While the invention has been particularly shown and
described with reference to preferred embodiments thereof,
15 it will be understood by those skilled in the art that the
foregoing and other modifications or changes in form and
details may be made therein without departing from the
spirit and scope of the invention.

   What is claimed is:

<u>CLAIMS</u>   32

1. A composition for use in the assay of vitamin B_{12} (cobalamin) wherein the active component is substantially specific to vitamin B_{12} and substantially free of substances which react with vitamin B_{12} analogues.

2. The composition of claim 1 wherein the active component is a binding protein.

3. The composition of claim 2 wherein the binding protein is intrinsic factor.

4. The composition of claim 3 wherein the intrinsic factor is selected from the group consisting of human IF, hog IF and rabbit IF.

5. The composition of claim 4 wherein the intrinsic factor includes human IF.

6. The composition of claim 4 wherein the intrinsic factor includes hog IF.

7. The composition of claim 1 wherein one or more protein binder which is non-specific to vitamin B_{12} is present and has been rendered substantially inactive or inoperative as to its ability to bind with vitamin B_{12} by treatment with a vitamin B_{12} analogue, said analogue being substantially reactive with said non-specific protein and substantially non-reactive with the material which is a specific binder for B_{12}, said analogue being present in an amount sufficient to render substantially all of the non-specific binder inactive or inoperative in its ability to bind with vitamin B_{12}.

8. The composition of claim 7 wherein the specific active binder includes intrinsic factor protein and the non-specific protein binder includes an R protein.

9. The composition of claim 8 wherein a portion of the vitamin B_{12} analogue is selected from the group consisting of cobinamide, CN-Cbl(bde-OH) and (3,5,6-Me3BZA) (CN,OH) Cba.

10. The composition of claim 7 wherein the vitamin B_{12} analogue is present in the amount of about a 10-fold to about a 100,000-fold excess, based on the molar amount of the non-specific protein.

11. The composition of claim 9 wherein the vitamin B_{12} analogue is present in the amount of about a 1-fold to about a 10,000,000-fold excess based on the molar amount of the non-specific protein.

12. The method of preparing the binder of claim 2 wherein a composition including binding material substantially specific to vitamin B_{12} and binding materials substantially non-specific to vitamin B_{12} and including a protein is treated with vitamin B_{12} analogues to remove substantially all of the non-specific vitamin B_{12} binding material, said vitamin B_{12} analogue being substantially reactive with said non-specific binder and substantially non-reactive with the material which is a specific binder for B_{12}, said analogue being added in an amount sufficient to render substantially all of the non-specific binder inactive or inoperative in its ability to bind with vitamin B_{12}.

13. The method of preparing the binder of claim 7 wherein a composition including protein binding material substantially specific to vitamin B_{12} and binding material substantially non-specific to vitamin B_{12} is treated with vitamin B_{12} analogue to render the binder non-specific to vitamin B_{12} substantially inactive or inoperative as to its ability to bind with vitamin B_{12}, said vitamin B_{12} analogue being substantially reactive with said non-specific binder and substantially non-reactive with the material which is a specific binder for B_{12}, said analogue being added in an amount suffi-cient to render substantially all of the non-specific binder inactive or inoperative in its ability to bind with vitamin B_{12}.

14. The method of claim 13 wherein the specific active binder includes intrinsic factor protein and the non-specific protein binder includes an R protein.

15. A radioisotope dilution assay for measuring the vitamin B_{12} level in a sample comprising: contacting said sample with a known amount of a radioisotope of vitamin B_{12} and a composition containing a binding protein substantially specific to vitamin B_{12}, said composition being substantially free of substances which bind vitamin B_{12} analogues.

16. The method of claim 15 wherein the binding protein is intrinsic factor.

17. The method of claim 15 wherein the binding protein consists essentially of protein selected from the group consisting of human IF, hog IF and rabbit IF.

18. The method of claim 15 wherein the source of vitamin B_{12} is selected from the group consisting of mammalian tissue and mammalian blood.

19. The method of claim 15 wherein the source of vitamin B_{12} is human blood.

20. The method of measuring vitamin B_{12} analogues present in a sample which also includes vitamin B_{12}, including the steps of:

assaying a portion of the sample with a binder which is a binder for both vitamin B_{12} and vitamin B_{12} analogues;

assaying a portion of the sample with a binder which is substantially specific to vitamin B_{12} and substantially free of substances which bind vitamin B_{12} analogues; and

then determining the difference between the two assays as being substantially indicative of the amount of vitamin B_{12} analogue in the sample.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL. G01N 33/16; U.S. CL. 23/230B, 230.3, 230.6; 252/408; 424/1, 1.5, 96

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
US	23/230B, 230.3, 230.6; 195/2; 252/408; 424/1, 1.5, 96

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category*	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	US,A, 3,434,927 Published 25 March 1969, Highley et al.	1-11
X	US,A, 3,442,819 Published 06 May 1969, See Examples I-III, Herbert.	15-20
X	US,A, 3,937,799 Published 10 Feb. 1976, Lewin et al.	15-20
X	US,A, 3,591,678 Published 06 July 1971, Ellenbogen et al.	1-11
A	US,A, 2,892,754 Published 30 June 1959, Nimeguen.	1-11
A	US,A, 2,912,360 Published 10 Nov. 1959, Baum et al.	1-11
A	US,A, 3,980,764 Published 14 Sept. 1976, Adams.	1-11
A,P	US,A, 4,133,951 Published 09 Jan. 1979, Charlton et al.	1-11
A,P	US,A, 4,136,159 Published 23 Jan. 1979, Stone.	1-11
A,P	US,A, 4,146,602 Published 27 March 1979, Gutcho et al.	1-11
A	GB,A, 771,068 Published 27 March 1957.	1-11
A	GB,A, 791,186 Published 26 Feb. 1958.	1-11
A	GB,A, 900,459 Published 04 July 1962.	1-11

* Special categories of cited documents: ¹⁵
"A" document defining the general state of the art
"E" earlier document but published on or after the international filing date
"L" document cited for special reason other than those referred to in the other categories
"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but on or after the priority date claimed
"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention
"X" document of particular relevance

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²
10 July 1979	17 JUL 1979

International Searching Authority ¹	Signature of Authorized Officer ²⁰
ISA/US	SIDNEY MARANTZ

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